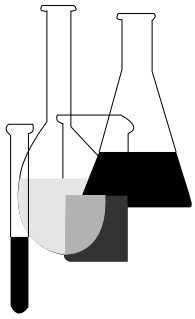
United States
Environmental Protection
Agency

Prevention, Pesticides
and Toxic Substances
(7101)

EPA 712–C–98–230
August 1998

Health Effects Test Guidelines

OPPTS 870.5550 Unscheduled DNA Synthesis in Mammalian Cells in Culture



INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations.

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD).

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U. S. Environmental Protection Agency under the Toxic Substances Control Act (15 U.S.C. 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U.S.C. 136, *et seq.*).

Final Guideline Release: This guideline is available from the U.S. Government Printing Office, Washington, DC 20402 on disks or paper copies: call (202) 512–0132. This guideline is also available electronically in PDF (portable document format) from EPA's World Wide Web site (http://www.epa.gov/epahome/research.htm) under the heading ''Researchers and Scientists/Test Methods and Guidelines/OPPTS Harmonized Test Guidelines.''

OPPTS 870.5550 Unscheduled DNA synthesis in mammalian cells in culture.

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, *et seq.*) and the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601).

(2) **Background.** The source materials used in developing this harmonized OPPTS test guideline are OPPT 40 CFR 798.5550 Unscheduled DNA synthesis in mammalian cells in culture and OECD guideline 482 Genetic Toxicology: DNA Damage and Repair, Unscheduled DNA Synthesis in Mammalian Cells in Vitro.

(b) **Purpose.** Unscheduled DNA synthesis (UDS) in mammalian cells in culture measures the repair of DNA damage induced by a variety of agents including chemicals, radiation and viruses. UDS may be measured in both *in vitro* and *in vivo* systems.

(c) **Definition.** The definitions in section 3 of TSCA and in 40 CFR Part 792—Good Laboratory Practice Standards (GLP) apply to this test guideline. The following definition also applies to this test guideline.

Unscheduled DNA synthesis in mammalian cells in culture is the incorporation of tritium-labeled thymidine (^{3}H-TdR) into the DNA of cells which are not in the S phase of the cell cycle.

(d) **Reference substances.** These may include, but need not be limited to, 7,12-dimethylbenzanthracene, 2-acetylaminofluorene, 4-nitroquinoline oxide or *N*-dimethylnitrosamine.

(e) **Test method**—(1) **Principle.** Mammalian cells in culture, either primary cultures of rodent hepatocytes or established cell lines, are exposed to the test agent. Established cell lines are treated both with and without metabolic activation. UDS is measured by the uptake of ^{3}H-TdR into the DNA of non-S phase cells. Uptake may be determined by autoradiography or by liquid scintillation counting (LSC) of DNA from treated cells.

(2) **Description**—(i) **Autoradiography.** For autoradiography, coverslip cultures of cells are exposed to test chemical in medium containing ^{3}H-TdR. At the end of the treatment period, cells are fixed, dipped in autoradiographic emulsion, and exposed at 4 °C. At the end of the exposure period, cells are stained and labeled nuclei are counted either manually or with an electronic counter. Established cell lines should be treated both with and without metabolic activation.

(ii) **LSC determinations**. For LSC determinations of UDS, confluent cultures of cells are treated with test chemical both with and without metabolic activation. At the end of the exposure period, DNA is extracted from

the treated cells. Total DNA content is determined and extent of ^{3}H-TdR incorporation is determined by scintillation counting.

(3) **Cells**—(i) **Type of cells used in the assay.** (A) A variety of cell lines or primary cell cultures, including human cells, may be used in the assay.

(B) Established cell lines should be checked for *Mycoplasma* contamination and may be periodically checked for karyotype stability.

(ii) **Cell growth and maintenance**. Appropriate culture media and incubation conditions (culture vessels CO_2 concentration), temperature, and humidity should be used.

(4) **Metabolic activation**. (i) A metabolic activation system is not used with primary cultures of rodent hepatocytes.

(ii) Established cell lines should be exposed to test substance both in the presence and absence of an appropriate metabolic activation system.

(5) **Control groups**. Concurrent positive and negative (untreated and/or vehicle) controls both with and without metabolic activation as appropriate should be included in each experiment.

(6) **Test chemicals**—(i) **Vehicle**. Test chemicals and positive control reference substances may be prepared in culture media or dissolved or suspended in appropriate vehicles prior to treatment of the cells. Final concentration of the vehicle should not interfere with cell viability or growth rate.

(ii) **Exposure concentrations**. Multiple concentrations of test substance, based upon cytotoxicity and over a range adequate to define the response, should be used. For cytotoxic chemicals, the first dose to elicit a cytotoxic response in a preliminary assay should be the highest dose tested. Relatively insoluble compounds should be tested up to the limits of solubility. For freely soluble nontoxic chemicals, the upper test chemical concentration should be determined on a case by case basis.

(f) **Test performance**—(1) **Primary cultures of rodent hepatocytes**. Freshly isolated rodent hepatocytes should be treated with chemical in medium containing ^{3}H-TdR. At the end of the treatment period, cells should be drained of medium, rinsed, fixed, dried, and attached to microscope slides. Slides should be dipped in autoradiographic emulsion, exposed at 4 °C for an appropriate length of time, developed, stained, and counted.

(2) **Established cell lines**—(i) **Autoradiographic techniques**. The techniques for treatment of established cell lines are the same as those for primary cultures of rodent hepatocytes except that cells must not enter S phase prior to treatment. Entry of cells into S phase may be blocked by several methods (e.g., by growth in medium deficient in arginine or

low in serum or by treatment with chemical agents such as hydroxyurea). Tests should be done both in the presence and absence of a metabolic activation system.

(ii) **LSC measurement of UDS**. Prior to treatment with test agent, entry of cells into S phase should be blocked as described in paragraph (f)(2)(i) of this guideline. Cells should be exposed to the test chemical in medium containing ^{3}H-TdR. At the end of the incubation period, DNA should be extracted from the cells by hydrolysis with perchloroacetic acid or by other acceptable methods. One aliquot of DNA is used to determine total DNA content; a second aliquot is used to measure the extent of ^{3}H-TdR incorporation.

(3) **Acceptable background frequencies**—(i) **Autoradiographic determinations**. Net incorporation of ^{3}H-TdR into the nucleus of solvent treated control cultures should be less than 1.

(ii) **LSC determinations**. Historical background incorporation rates of ^{3}H-TdR into untreated established cell lines should be established for each laboratory.

(4) **Number of cells counted**. A minimum of 50 cells per culture should be counted for autoradiographic UDS determinations. Slides should be coded before being counted. Several widely separated random fields should be counted on each slide. Cytoplasm adjacent to the nuclear areas should be counted to determine spontaneous background.

(5) **Number of cultures**. Six independent cultures at each concentration and control should be used in LSC UDS determinations.

(g) **Data and report**—(1) **Treatment of results**—(i) **Autoradiographic determinations.** For autoradiographic determinations, once untransformed data are recorded, background counts should be subtracted to give the correct nuclear grain count. Values should be reported as net grains per nucleus. Mean, median, and mode may be used to describe the distribution of net grains per nucleus.

(ii) **LSC determinations**. For LSC determinations, ^{3}H-TdR incorporation should be reported as disintegrations per minute per microgram of DNA. Average disintegrations per minute per microgram of DNA with standard deviation or standard error of the mean may be used to describe distribution of incorporation in these studies.

(2) **Statistical evaluation**. Data should be evaluated by appropriate statistical methods.

(3) **Interpretation of results.** (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the incorporation of ^{3}H-TdR into treated cells. Another

criterion may be based upon detection of a reproducible and statistically significant positive response for a least one of the test points.

(ii) A test substance which does not produce either a statistically significant dose-related increase in the incorporation of ^{3}H-TdR into treated cells or a statistically significant and reproducible positive response at any one of the test points is considered not to induce UDS in the test system.

(iii) Both biological and statistical significance should be considered together in the evaluation.

(4) **Test evaluation.** (i) Positive results in the UDS assay indicate that under the test conditions the test substance may induce DNA damage in cultured mammalian somatic cells.

(ii) Negative results indicate that under the test conditions the test substance does not induce DNA damage in cultured mammalian somatic cells.

(5) **Test report.** In addition to the reporting recommendations as specified under 40 CFR part 792, subpart J, the following specific information should be reported:

(i) Cells used, density and passage number at time of treatment, number of cell cultures.

(ii) Methods used for maintenance of cell cultures including medium, temperature, and CO_2 concentration.

(iii) Test chemical vehicle, concentration, and rationale for selection of concentrations used in the assay.

(iv) Details of both the protocol used preparation of the metabolic activation system and its use in the assay.

(v) Treatment protocol.

(vi) Positive and negative controls.

(vii) Protocol used for autoradiography.

(viii) Details of the method used to block entry of cells into S phase.

(ix) Details of the methods used for DNA extraction and determination of total DNA content in LSC determinations.

(x) Historical background incorporation rates of ^{3}H-TdR in untreated cell lines.

(xi) Dose-response relationship, if applicable.

(h) **References.** The following references should be consulted for additional background material on this test guideline.

(1) Ames, B.N. et al. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. *Mutation Research* 31:347–364 (1975).

(2) Rasmussen, R.E. and Painter, R.B. Radiation-stimulated DNA synthesis in cultured mammalian cells. *Journal of Cell Biology* 29:11–19 (1966).

(3) Stich, H.F. et al. DNA fragmentation and DNA repair as an in vitro and in vivo assay for chemical procarcinogens, carcinogens and carcinogenic nitrosation products, Screening tests in chemical carcinogenesis. Eds. Bartsch, H., Tomatis, L. IARC Scientific, Lyon, No. 12 (1976) pp. 617–636.

(4) Williams, G.M. Carcinogen-induced DNA repair in primary rat liver cell cultures: a possible screen for chemical carcinogens. *Cancer Letters* 1:231–236 (1976).

(5) Williams, G.M. Detection of chemical carcinogens by unscheduled DNA synthesis in rat liver primary cell cultures. *Cancer Research* 37:1845–1851 (1977).